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Red cell investigations: Art and artefacts

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Abstract

Investigations of red blood cells include a wide range of methodologies ranging from population measurements with a billion cells evaluated simultaneously to single-cell approaches. All methods have a potential for pitfalls, and the comparison of data achieved by different technical approaches requires a consistent set of standards.
Here, we give an overview of the common methodological mistakes and how to avoid them. Additionally, we propose a number of standards that we believe will allow for data comparison between the different techniques and different labs. Here, we consider biochemical analysis, flux measurements, flow cytometry, patch-clamp measurements and dynamic fluorescence imaging as well as emerging single-cell techniques, such as the use of optical tweezers and atomic force microscopy.

1 Introduction

Contrary to a common belief, the red blood cell (RBC) is a cell type that is neither simple, nor easily obtainable in a pure form. Yet, it is probably the most studied cell type in the history of the life sciences starting with the microscopic observations of Jan Swammerdam in approximately 1660[1]. Nevertheless, as in most other fields of science, contradictory data are common. Sometimes it is possible to unify initially opposing results, e.g., reconciling reports on the electrogenic permeabilities in malaria-infected RBCs[2,3] or on the isolation of lipid rafts from RBCs[4-6]. In other cases, specific issues have not been completely resolved, for example, the number of Gardos channels per RBC[7,8] or contradictory data regarding prostaglandin E_2-induced cation fluxes[9,10,11].

However, discrepancies often originate from different experimental protocols, inducing different or even opposing degrees of artefacts. Sometimes, artefacts may lead to completely wrong conclusions. This is a serious problem, as revealed in a recent publication[12] in Nature. Here, a standard method intended for the isolation of mononuclear cells (MNCs), based on the density-gradient centrifugation of blood, was mistakenly used to isolate RBCs in an allegedly pure form. The entire paper is affected by this artefact, but it obviously passed the review process in one of the most prestigious journals.

To avoid this and other common artefacts, as well as to establish a basis for good laboratory practices in RBC research, a subgroup of the European Red Cell Society (ERCS) was formed to initiate standards for a better inter-methodological as well as inter-laboratory comparison of RBC-derived data. As an initial attempt, here, we present the first "guidelines" for avoiding artefacts in RBC research: In the first part, we discuss the general challenges, such as obtaining pure RBC preparations, experimental conditions in general and the comparison of studies between different species. In the second part, we consider some of the most popular methods in RBC research, discussing possible pitfalls, how to avoid them and the conditions for comparing/combining different methodologies.

Our hope is that this report will be useful to those approaching the study of RBCs, to avoid stumbling into major artefactual conditions and obtaining the best results from the experiments.

2 General considerations

2.1 Obtaining pure cell preparations

The vast majority of biochemical studies, but also all other types of cell population measurements, have been carried out, and still are, using bulk suspensions of supposedly pure RBCs. The RBCs are obtained by sedimenting the cells by centrifugation from a sample of whole blood that has been “washed” with variants of a physiologic solution, followed by removal of the supernatant and the thin superficial layer of cells. The latter, the so-called “buffy-coat”, is indeed enriched in white blood cells (WBCs), or leukocytes, but these cells belong chiefly to the MNC type, i.e., lymphocytes and monocytes. The most abundant WBCs, however, the polymorphonuclear neutrophil granulocytes (PMNs), tend to remain mixed with the RBCs owing to the similar density of the two cell types, contaminating the RBC sample[6]. The only way of removing most of the WBCs is by filtering the blood with leukodepletion filters. Roughly speaking, if the total content of PMNs per million RBCs is 1000 in whole blood, it will decrease, at best, to 100 in washed blood and to <10 in filtered blood[6].

A simple and reliable procedure for RBC purification that is suitable for samples of small volumes and easy to implement in every lab is filtration through cellulose, as was originally proposed by Beutler et al.[13] and described in detail in the supplementary material of Achilli et al. [14].

We propose this simple concept as a standard method and good laboratory practice in

RBC research. It should be emphasised, however, that filtration might not be applicable in all instances, e.g., for pathological RBCs, because its functioning principle appears to be based largely on the difference in deformability between RBCs and WBCs[15]. The latter are much less deformable than normal RBCs and are therefore retained in the filter for a longer time than RBCs. However, in certain RBC pathologies, RBC deformability is abnormally reduced, and this may result in reduced filterability (hereditary spherocytosis, hereditary elliptocytosis, ovalocytosis, sickle cell anaemia). The task of quantifying low WBC levels is by no means a simple one, and special techniques have been devised for this purpose. As a general remark, microscope counting using conventional haemocytometer chambers is impractical and not sensitive enough. The flow cytometry (FCM) approach is meaningful only if the number of total events counted in each analysis is sufficiently high to reveal 1 WBC per 10^6 RBCs, which implies long analysis times[16]. An extremely sensitive and inexpensive method for the quantification of PMNs in blood samples that can be easily implemented in all labs is the technique of gelatin zymography, as recently adapted[14].

Typical artefacts

The consequences of having a PMN-contaminated RBC suspension can be deleterious. Two main types of artefacts can results from such a situation: (i) attribution to the RBCs of a component/function that in fact belongs to the PMNs; (ii) damage to RBCs resulting from hydrolases and oxidases released by activated or broken PMNs.

The first issue has already been exemplified in the introduction. As shown in Figure 1A, the method results in the isolation of a fraction of RBCs together with all the PMNs that were originally present in the blood sample, without even reducing the number of PMNs, as would occur if a conventional centrifugation-based wash of the blood and removal of the “buffy-coat” were performed.

The artefactual results that originate from PMN hydrolases damaging RBC components are exemplified by the controversy on the isolation and characterisation of lipid rafts from RBCs[6]. The most powerful and constitutively active hydrolases in the PMNs are the serine proteases elastase and cathepsin G. These hydrolases are normally confined at high concentrations in cytoplasmic vesicles (granules) and only released upon cell activation. Detergents can easily free the proteases from the granules. It was shown that even the presence of one PMN per million RBCs is able to release enough proteolytic power to damage, if not fully inhibit, highly sensitive RBC proteins such as ankyrin and protein 4.1[6].

Another common situation that could give rise to artefactual results is the preparation of “ghosts” from RBCs by hypotonic haemolysis. If the RBCs are contaminated by PMNs and the buffers used are not effectively supplemented with anti-proteases, the RBC membrane proteins will almost certainly be damaged (Figure 1B,C). The workaround to this problem is the filtration of the blood and the use of freshly prepared lysing buffers containing a working concentration of anti-proteases[17,18].

2.2 Experimental conditions

Other factors that must be standardised to be able to compare the obtained data

between different laboratories are the temperature, shear stress, medium content, especially traces of serum, and the condition of cells used in the experiments. Furthermore, recent studies emphasise the importance of co-factors and substrates of several receptors, which may contribute to the experimental outcome. Those include the presence of prostaglandins, hormones, amino acids and other natural or pathological components of blood plasma.

Possible artefacts and their causes

(i) Temperature-related artefacts include ion misbalance and the following changes in cell volume and Ca^{2+}-dependent processes. Temperature sensitivity depends on the particular approach, but it can be severe, differing, e.g., between different types of ion transporters. The decrease in the activity of ion transporters with a decrease in temperature by 10 degrees (Q10) is approximately 30-fold for the Ca^{2+}-ATPase[19], approximately 3-fold for the Na^+/K^+-ATPase[20] and approximately 1.5-3-fold for most of the ion transporter systems[21,22]. Thus, temperature changes may have a pronounced effect on the intracellular Ca^{2+} levels and the Na^+/K^+ distribution. The temperature may not be fixed at 37°C in particular experimental settings (e.g., controlling the temperature can be complicated for patch-clamp investigations). However, temperature as a factor has to be taken into account, and the potential side effects must be controlled.

(i) Serum and the multiple biologically active factors it contains, including albumin and factors bound to it, such as interleukins, prostaglandins, insulin and amino acids, can introduce artefacts. Depending on the experimental settings, investigations are conducted in serum-containing or serum-free media. Proteins introduced with serum have been shown to play an active role in regulating the activity of ion transporters in RBCs obtained from healthy and diseased subjects. Little is known about the serum components mediating the effects. It has been shown that PGE_2 activates Ca^{2+} uptake by RBCs[10,23]. Insulin interacts with its receptors, inducing activation of eNOS in RBCs[24]. From studies on malaria-infected cells, it is now well recognised that traces of serum change the membrane conductance upon infection[2,25]. Nevertheless, such a phenomenon may also be observed when performing experiments on uninfected cells[26]. This leads to the conclusion that serum-proteins play a role in modulating the activity of transport proteins[27]. This is a potential source of discrepancy between single cell and bulk measurements. In most of the latter, at least serum albumin is present (usually 5%) as a supplement in the suspending medium. The presence of several amino acids in the incubation medium makes a substantial difference in the response of cells to oxygen, insulin and erythropoietin stimulation. Among these amino acids are L-arginine, which is a substrate for endothelial nitric oxide synthase[18], and the N-methyl D-aspartate receptor agonists glutamate and glycine, as well as homocysteine, which stimulates Ca^{2+} uptake by human and rat RBCs[28]. Treatment of RBCs with relatively high concentrations of orthovanadate in the presence of 1-2 mM extracellular Ca^{2+} results in irreversible pathological alterations of cell morphology, followed by blebbing and finally the loss of membrane integrity, particularly at room temperature when the Ca^{2+} pump function is reduced (Figure 2A). This often remains unnoticed when working with RBC suspensions.

(ii) Intercellular differences originating from storage (fresh cells vs. stored cells and storage conditions), inter-individual and inter-cellular variability are sources of artefacts. Often, stored/conserved RBCs are used for measurements. RBC preservation media are Ca^{2+}-free, low in Na^{+} and enriched with K^{+} and glucose. RBC preservation results in gradual adenosine triphosphate (ATP) and 2,3-bisphosphoglycerate deprivation and oxidation of glutathione, which begin after one day of storage (Figure 2B). Replacement of the storage medium with Ca^{2+}-containing plasma-like medium (1.8 mM $CaCl_2$, 150 mM NaCl, 4 mM KCl, 5 mM glucose) results in the events illustrated in Figure 2B. The cells will shrink due to acute Ca^{2+} overload, and further ATP deprivation occurs due to acute activation of the Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase caused by acute Na^{+} and Ca^{2+} overload. The results obtained using such cells may hardly be compared with those obtained from fresh RBCs. Restitution of stored cells may be useful for avoiding storage-induced artefacts. Preconditioning of stored blood (rejuvenation) has been proposed[29], and the corresponding "Rejuvenation Solution" (Rejuvesol; enCyte Systems, Inc., Braintree, Mass) containing phosphate, inosine, pyruvate, and adenine, or 15 mM D-ribose was shown to be beneficial when applied before the transfusion[30]. Because the components of rejuvenation solutions actively interfere with intracellular metabolism and the redox state, we propose to use a "minimally invasive" preconditioning protocol. The stored cells are re-suspended in the incubation medium of interest in the presence 0.5 mM Ca^{2+}, 10 mM glucose and 0.1% BSA at room temperature one hour prior to the experiment. This time is required to restore the activity of the Ca^{2+} pump at a sub-physiological temperature and to provide substrates for glycolytic enzymes.

Most artefacts arise from the lack of attention to these factors. The composition of incubation media varies markedly between experiments. The impact of oxidation, methaemoglobinemia, phospatidyl serine (PS) exposure and other membrane-related events, as well as that of the addition of ion transport inhibitors (e.g., vanadate often present during Ca^{2+} uptake measurements, see Figure 2A), on the cell morphology, ion content, redox state and metabolic status may be dramatic, but it has rarely been taken into account.

The redox status of the cells is an important parameter to control. Oxidation has a profound effect on metabolism, regulation of cell volume, and cytoskeletal structure. Reducing cell deformability induces Ca^{2+} entry, leading to PS exposure, membrane blebbing and eventually premature cell death[31]. Nevertheless, it was also shown that oxidation may activate anion channels, mimicking pathways that are activated upon malaria infection[32,33]. Even if the threshold seems to be rather high, the oxidation level might be high enough in some cells to trigger artificial responses in some protocols. Most importantly, throughout their lifetime, RBCs are continuously exposed to high oxidative stress. Oxidative defence capacities may decrease with RBC aging[34], and senescent RBCs show alterations (e.g., increased denaturation of haemoglobin, membrane binding of hemichromes and free iron, aggregation of band 3 protein, deposition of antibodies and complement fragments, PS exposure) similar to those of oxidised cells[35,36].

Facilitated ageing occurring under conditions of sheer stress (e.g., in patients with polycythaemia) is also associated with oxidative stress[37]. Furthermore, storage of RBCs

results in progressive oxidative stress and loss of reduced glutathione along with ATP deprivation. For that reason experimental observations obtained using RBCs from a blood bank may differ significantly from those generated using freshly withdrawn blood. Further support comes from whole-cell patch-clamp experiments reporting that oxidation induced anion selective currents[32,38,39].

Sufficient levels of glucose, a lack of Ca^{2+} overload and sheer stress are essential for maintenance of the citrate-containing glutathione pool. Recent studies revealed that some plasma components are required for endothelial NO synthase to function. L-arginine (100-300 μM) and nitrite (~150 nM in human plasma) are essential for maintenance of NO production by RBCs under normoxic and hypoxic conditions, respectively[24,40], and their absence in the incubation medium, as well as manipulation of the intracellular Ca^{2+} levels, will result in uncoupling of NO production and progressive oxidative stress, especially when the treatment includes manipulation of oxygen levels or activation of NO synthase[18,28,41].

Another example of the importance of the ionic composition of the incubation media arises from patch-clamp measurements of malaria-infected RBCs: Whereas at physiological saline concentrations, at least two different types of anion channel activity can be described, when supraphysiological concentrations of Cl^- are used[42,43], one of the channels has (i) a saturated single conductance and (ii) an open probability close to zero above the threshold chloride concentration[3]. This last phenomenon explains the majority of the discrepancies reported in the field, and it is tempting to think that the same limitation may apply to uninfected RBCs.

2.3 Interspecies studies

The challenge of how to compare studies performed in different species is widespread in biomedical science. The power of genetic manipulation in combination with the short generation cycle makes mice an increasingly popular animal model. Obvious advantages often overwhelm concerns about the reliability of results derived from animal models of human diseases. This problem also applies to RBC research and originates from the fact that the basic characterisation of mouse RBCs is rather limited. Before the advent of transgenic animals, mice were not a particularly widespread model for studying RBCs.

Potentials of comparative studies

Comparative RBC research continues to build on species-specific studies involving, e.g., domestic animals. In this field, a substantial number of publications and even textbooks are available[44,45]. Sometimes, the switch to animal RBCs may provide invaluable advantages over human RBCs. These advantages might be such simple properties as the cell size. For instance the amphiuma RBCs have an elliptical size of ~62 µm in length and ~36 µm in width and are used to perform the initial potential measurements in RBCs[46]. The RBCs of fish (6.5-44.6 μm diameter), amphibians (16-70 μm) and birds (9.7-15.4 μm) contain organelles such as a nucleus, mitochondria and ribosomes. These qualitative differences compared to human RBCs might be advantageous or disadvantageous and can be used as experimental tools.

The great variations in RBCs between species on the one hand and a broad conservation on the other hand allows the use of animal RBCs as particular models for certain protein manipulations, even in the organelle-free mammalian RBCs, that would otherwise require the breeding of transgenic animals. Examples include the RBCs of carnivora that lack the Na^+/K^+- pump[47] (instead, they have a Na^+/Ca^{2+} exchanger, which is absent in the RBCs of other species) or sheep RBCs that do not seem to contain scramblase[48]. There is list of differences[49] that cannot be covered in this paper – furthermore, the protein and lipid distributions of RBCs between species can differ considerably[50,51].
Thus, vast amounts of information on the alternative models that may be used to study the pathological alterations in human RBCs are not used. Making the results of comparative studies on RBCs more “visible” will help to acknowledge the advantages that these cells provide.
Knowing all these differences, it should be a habit of good laboratory praxis (as well as reviewing praxis) to either perform studies (publications) just within a defined species or, when mixing species, to show - whenever possible - explicitly the transferability of the "previous step", at least in the supplemental material. This rule of course needs to be adapted if the animal model is used as a "modified source" of RBCs.

3 Methodological considerations

3.1 Proteomics

Proteomics is most likely the method that is most affected by contamination of cell preparations, as outlined in 2.1, Obtaining pure cell preparations. This holds true because proteomic studies are still carried out on cell suspensions, although single-cell approaches have been introduced[52].
The importance of the pure cell preparations is efficiently and impressively illustrated by some of the most recent proteomic studies, where care was taken to reduce WBC contamination of the RBCs, resulting in a list of less than 300 recognised RBC membrane proteins[53], compared to the much larger number of supposedly erythrocytic proteins presented in earlier catalogues.
Presently, the proteomic studies of RBCs are still somewhat separated from functional studies, resulting in protein catalogues that do not (yet) fit with functional identified proteins from, e.g., patch-clamp recordings. Bridging this gap will be one of the challenges of future RBC research.

3.2 Ion fluxes

Measurements of ion fluxes through the RBC membrane are performed using various approaches. Radioactive tracers have been used for unidirectional flux measurements for many decades. This technique allows quantification of unidirectional movements of ions by electroneutral and electrogenic ion transporters as well as residual ion fluxes. Other methods to assess ion movements through the membrane are based on

monitoring of net ion uptake/loss by means of ion-selective electrodes, flame photometry, atomic absorption spectrophotometry, etc.

Accumulation or loss of radioactive tracers may be estimated with high sensitivity (up to single disintegration events) using beta- and gamma-counters. For most ions, the corresponding radionuclides, which may play a role as isotopic carriers, have relatively long half-lives (weeks to months). Rubidium-86 ($T_{1/2}$=18.6 d) is often used as a tracer because the most suitable $^{42}K^+$ radionuclide has a rather short half-life ($T_{1/2}$=12.5 h) and requires a supply for fresh radioisotopes, e.g., the proximity of a cyclotron to the lab where the ion fluxes are assessed. With some rare exceptions[54], discrimination between K^+ and Rb^+ by ion transport systems in RBCs does not exceed 20%[55].

Pitfalls and limitations

For monitoring the kinetics of the radioactive tracer distribution, one may assess the unidirectional inward and outward fluxes as well as a steady-state distribution of selected ion species between the cell and the medium, considering that the cell lacks compartmentalisation. If this is not the case, as for intracellular Ca^{2+}, in RBCs of patients with sickle cell disease[56], cytosolic free Ca^{2+} cannot be estimated from the $^{45}Ca^{2+}$ distribution between the cells and the medium. Most of the Ca^{2+} in that case is accumulated in the intracellular inside-out vesicles that are most likely enriched with Ca^{2+} pumps[57], and an increase in the intracellular $^{45}Ca^{2+}$ levels is not always followed by the activation of Ca^{2+}-sensitive K^+ (Gardos) channels.

All discussed, measurements of ion fluxes bear a common limitation: flux measurements are performed in suspension, and the considerations discussed in 2.1, Obtaining pure cell preparations, apply. So far, studies to assess the role of WBC and platelet contamination in possible artefact generation when measuring ion fluxes using radioactive tracers are lacking.

Another point that is seldom taken into account is the effect of the electro-neutrality of compartments on ion movements. Cation movements, such as those mediated by Gardos channel activity, that lead to cell dehydration are known to be rate limited by anion movements. In many cell suspension experiments, thiocyanate (SCN^-) is used to bypass this limitation of anion movements. Ten millimolar is usually sufficient to saturate this effect[58], avoiding important changes in the isoelectric point of impermeant anions and RBC hydration that are observed at higher SCN^- concentrations[59]. Apart from ion flux experiments, this could also apply to patch-clamp experiments aiming to investigate cation channel activity. Even if this consideration does not apply in the whole-cell configuration because the anion supply is provided by the pipette content, it can impair the movement of cations in the cell-attached configuration. In this case, run-down of channel activity might be observed and conclusions can be drawn erroneously.

3.3 Patch-clamp

During the past three decades, electrophysiological studies have revealed that the human RBC membrane is endowed with a large variety of ion channels[60-64]. However, their physiological role remains widely unclear; they barely participate in the RBC homeostasis, which is based on an almost total absence of cationic permeability and

minute anionic conductance[65]. Nevertheless, due to the pioneering work of Hamill on human and frog RBCs[58,66], the patch-clamp technique applied to RBCs has proven to be a powerful method to decipher the involvement of ionic conductances mainly in pathophysiological scenarios[32,42,59,62,67-69].

Challenges to patch-clamp RBCs

The main problem when attempting to perform patch-clamp on RBCs lies in the small size of the cells, which especially holds true for mammalian RBCs (*2.1-9.4 μm*) (cp. 2.3, Interspecies studies). This small size imposes four major challenges:

(i) The opto-mechanical properties of the hardware require high-quality microscopes and at least 20x objectives. A 40x objective with phase or Nomarski contrast is usually necessary for recordings on malaria-infected cells to recognise the infected RBCs. When approaching the pipette to form a seal, very precise micromanipulators are required.

(ii) RBCs are "designed" for passing through small capillaries. When passing through the spleen, RBCs have to go through tiny slits whose mean size has been recently measured at 1.89 μm in length and 0.65 μm in width[43]. Therefore, patch-pipette tips must be rather thin, with an opening smaller than 1 μm (corresponds to roughly 10-15 MΩ in physiological saline solutions) to avoid the entry of the cell into the pipette. Besides the pipette size, its shape has to be adapted such that a piece of membrane enters the pipette for seal formation without totally entering into the pipette when depression (typically 20 mbar) is applied. The pipette tip must be thin enough, but at the same time tapered enough, to preserve a low R_{access} value (see (iii), below). Another issue arises from RBC's high deformability. The portion of the RBC membrane that enters into the pipettes during seal formation varies. Furthermore, it has been recognised that membrane deformation induces transient Ca^{2+} entry in RBCs[70]. Such transient activity may generate secondary transient anionic channel activity[71]. This phenomenon leads to a change in the intracellular K^+ concentration that has to be taken into account for data interpretation. Therefore, the time of seal formation and calibrated depression must be mentioned in publications.

(iii) Electrical issues have to be considered, notably for the whole-cell configuration. Figure 3 describes the electric model for the cell-attached configuration and the whole-cell configuration. Do the limitations described in Figure 3 apply to RBCs? From suspension experiments, the membrane resistance R_m of human RBCs was estimated to be in the range of 10^6 Ωcm², with chloride resistance R_{Cl} ranging between 10^5 and 10^6 Ωcm² [72]. Given a mean surface of 135 μm², the whole-cell resistance can be estimated to be between 10 and 40 GΩ. This is in the range of the seal values of patched RBCs and explains why whole-cell experiments were not used on RBCs for a long time[73]. The conductance was estimated to be too low to discriminate properly between channel activity and seal leakage. However, the membrane resistance can be considerably lower when the ion channels in the RBC membrane are activated. Anion conductance rises to several nS, e.g., in PKA-activated, oxidised or malaria-infected RBCs, and cation conductance can be triggered when cation channels are activated. Thus for whole cell experiments, R_m remains much lower than R_{seal}, and the current and voltage do not suffer high distortion. Another parameter to take into

account is the pipette access resistance R_{access}. Because the pipette tip must be rather narrow when patching RBCs, precautions must be taken. The R_{access} value is determined by the pipette geometry, solution composition and possibly by the presence of cell debris generated by membrane rupture. The use of pipettes immediately after pulling, their adapted shape, with rapidly tapering geometry, and the filtering of all pipette solutions with 0.2 μm filters helps to maintain low R_{access} values.

(iv) The small RBC size results in a small membrane capacitance of approximately 1-1.3 pF[74,75]. This becomes relevant during the transition from the cell-attached to whole-cell configuration. The rupture of the membrane fragment inside the pipette tip is typically achieved by a brief electrical pulse (200 ms, 500 mV). A successful whole-cell configuration can be checked via the sudden appearance of membrane capacitance transient currents, which can be easily compensated on the amplifier. Nevertheless, the situation is different in plate-based "pipettes" as they are used by automated patch-robots (cp. Figure 4). There, the basal capacitance of the plate is much higher and an increase of 1 pF is almost invisible. Therefore, the major indication for reaching the whole-cell state is the increase in current, which is a challenge because differentiation between the loss of seal resistance and the whole cell current needs to be probed in the experimental protocol. However, if the seal resistance is approximately 10 GΩ, the current leakage at +100 mV can be calculated to be 10 pA, presenting a relation to Ohm's law. Typical whole-cell recordings show current values between 200 to 1000 pA or even higher, which often are rectifying, i.e., the do not follow Ohm's law; then, the leak always remains below 1-5% of the total current.

The automated patch-clamp recordings were used to test for the heterogeneity of naive RBC conductance among the RBCs of a donor as well as investigating the variability between different donors. Figure 4 depicts such a comparison for 2 healthy donors.

The simultaneous measurements of 4 to 96 RBCs (depending on the model of the automated patch systems) allow for measurement of a population of RBCs with exactly the same experimental procedure, and there is no experimental bias towards choosing a (particular) cell. In contrast, classical patch-clamp allows for more (visual) control over the particular experiment/cell and at least an order of magnitude lower noise level, typically approximately 1 pA.

Comparison of Patch-clamp data with other entities

Comparing data from cell suspension experiments (cp. 3.2, Ion fluxes) and those issued from patch-clamp studies is a common but difficult task, which can be exemplified by the entry of Ca^{2+} observed in sickle cells upon deoxygenation. This entry, designated P_{sickle}, is best characterised as a poorly selective permeability pathway for small, inorganic monovalent and divalent cations[76]. Experiments in which the fraction of activated cells was studied as a function of the external Ca^{2+} concentration showed that sickling is a stochastic event of random intensity among HbSS RBCs, capable of generating maximal Gardos channel activation in a small fraction of cells during each deoxygenation-sickling pulse. Consistent with the stochastic nature of P_{sickle}, repeated pulses led to the progressive accumulation of dense cells, whereas a single long pulse caused only an early production of a single small fraction of dense RBCs[77]. Lew *et al.* eventually

depicted this nature clearly by writing: “When electrophysiologists finally approach the study of P_{sickle} under patch-clamp, they ought to bear in mind the probabilistic nature of P_{sickle} in each deoxygenation pulse before consulting their psychiatrist for the lack of reproducibility!” [76]

One has to keep in mind that electrophysiology conclusions are drawn from results where the membrane potential is changed at will by the experimenter, meaning that they are rarely obtained at the resting membrane potential, rendering comparison with cell suspensions difficult. This is exemplified in a recent study, where it was shown that increased membrane permeability for sorbitol in malaria-infected RBCs could not easily be reconciled with data from whole-cell experiments[78]. Indeed, in isosmotic sorbitol haemolysis, the membrane potential reaches values above +50 mV due to the absence of charges at the extracellular side of the membrane. Subsequent comparison of these data to that obtained with patch-clamp (at this membrane potential, inwardly rectified currents induced by infection are almost totally abolished[59,62]) seems impossible.

3.4 Flow cytometry

FCM is a technique that uses optical detection methods for counting and analysing particles in the size range of micrometres. In comparison to microscopic investigations of single RBCs (see below), the application of FCM and cell sorting present some advantages as well as disadvantages.

Advantages

A major advantage of FCM compared to single-cell imaging is the inherent analysis of a larger amount of cells within a shorter time (a minimum of several 10,000 cells vs. a few hundred cells). This reduces the statistical noise. The gating for cell populations is easy and reduces the analysed cells to a dedicated population out of a heterogeneous sample. The forward scatter mode shows the size distribution of the cells. Although it is by no means an exact measure of the absolute cell volume, it can be used as an indicator of the relative size changes of the RBC samples.

The side scatter mode shows the “granularity” of the cell, which is related to the complexity of structures in the cell interior. It can provide information on the presence of different cell types in a single suspension of cells (e.g., in blood). A useful feature of flow cytometry is connected with the possibility of measuring the fluorescence emitted by suitable fluorochromes that are used as probes for a given particular cell property. Fluorescently labelled antibodies and fluorescent probes sensitive for a particular chemico-physical parameter of the cell (e.g., pH, Ca^{2+}, PS exposure, mesomorphic state of the lipids) are the most commonly used fluorescent molecules.

Limitations

Due to the measurement technique, cells have to pass the cuvette in a high-speed fluid stream. This limits measurements to cells in a suspension and excludes larger aggregates. However, doublets of RBCs can be easily recognised by the fluorescence signal forward or side scatter. Although the side scatter is an indicator for the granularity and surface shape, it is not possible to measure and reliably distinguish the different

shapes (echinocytes, discocytes, stomatocytes) of RBCs. In the forward and the side scatter, RBCs present shapes that are nearly similar and overlapping signals.
The fluorescence intensities observed by FCM are integrated values of the entire cell and do not resolve a subcellular distribution of the fluorescence as in imaging (see below).
In some experiments, the formation of microvesicles can be observed. Due to the small size of the microvesicles, they will be shown in the forward and side scatter below the threshold together with the cell debris and dead cells and will normally be discarded. However, the fluorescence might be used to discriminate the vesicles from the debris, and this could allow a quantitative analysis.
In contrast to single-cell imaging approaches, it is not possible to follow the kinetics of any signal in a single cell. After measurement of the optical parameters, the cell is either discarded or collected in a tube with RBCs depicting the same properties.
In all fluorescence measurements of RBCs, haemoglobin shows a strong absorption of UV and visible light (for more details and discussion, see 3.5, Cellular Imaging). This requires dyes with emission wavelengths above 600 nm or brighter fluorophores (compared to thresholds that are known for haemoglobin-free cells).

Potential artefacts

Before measuring the different parameters, the cells are sucked under pressure in a fluid stream through a small capillary into the measurement chamber. While the cell suspension is passing through a capillary in the FCM to the measurement chamber, the cells can be exposed to shear stress because of the different speeds of the sample and the sheath fluid. An applied shear stress can induce different mechanisms such as the activation or inactivation of physiological processes in the cells (e.g., Ca^{2+} increase in RBCs exposed to mechanical stress[79]) or even damage the membrane. Values for the applied pressure can reach 500 kPa and higher (manufacturer information), which exceed the normal systolic arterial blood pressure value of approximately 15 kPa by a factor of more than 30. It is well known from other cell types that cell damage can occur because of the applied pressure[80], and our own observations showed that a population of fragile RBCs (observed in imaging) can disappear in FCM (unpublished observations).

3.5 Cellular Imaging

Live cell imaging is a popular method to explore cellular signalling[81]. However, for the investigation of RBCs, it is rather sparsely applied. This might be due to three major drawbacks:

(i) The absorption spectrum of the haemoglobin heavily interferes with the absorption of many commonly used dyes and additionally quenches their emission, as exemplified by the most popular Ca^{2+} fluorophores (Figure 5).
(ii) Due to the lack of a protein translation mechanism, the application of fluorescent proteins and genetically encoded biosensors as an emerging tool in biomedical research is limited to the generation of transgenic animals (cp. also [82]).
(iii) In live cell imaging (time laps), each experiment follows a substantial number of cells (not just one as, e.g., in patch-clamp recordings), and each cell is followed over

an arbitrary time course (not just one time point as, e.g., in FCM). This leads to a large amount of data. In light of the increasing awareness that RBCs can form a highly heterogeneous population (cp. Figure 4), there is a requirement for analysis guidelines that are not yet filed and that would exceed the size of this section.

Although these 3 points are serious and have to be taken into account, cellular imaging is a powerful tool in RBC research.

Potential artefacts and how to avoid them

A number of points have to be considered to avoid artefacts. In imaging approaches, dye molecules and photons are used to probe the cells. Photons can interact with the cellular constituents and may induce what is commonly referred to as phototoxicity. For RBCs, this is known for near infrared light[83] and for the interaction of UV light with haemoglobin, resulting in the generation of a highly fluorescent photoproduct, most likely bilirubin[84].

The interaction of the photons with the dye can lead to photobleaching and induce a "loss of signal". This decreased fluorescence leads to underestimation of the signal of interest. Furthermore, there is another almost opposite effect that is often neglected but may occur with some dyes, e.g., with Fluo-4, the so-called "antibleaching". This is, in this example, the light-mediated induction of Ca^{2+} insensitive but highly fluorescent dye molecules that can occur if illumination of high intensity is used. Consequently, the signal of interest is prone to be overestimated.

Additionally, triple interactions between endogenous proteins, fluorescent dyes and photons may alter the properties of the fluorescent read-out. If there is a binding affinity between the endogenous proteins and the fluorescent dye under certain conditions[85], Förster Resonance Energy Transfer (FRET) occurs and consecutively alters the fluorescence intensity, spectral properties and fluorescence lifetime. In RBCs, FRET can occur, e.g., between the dye Fura-red and haemoglobin (unpublished results).

It must be noted that FRET can also be used in a beneficial way, as nicely shown by Esposito et al. [86] for imaging the haemoglobin concentrations in malaria-infected RBCs.

Yet another factor that influences the fluorescence intensity is RBC volume changes because a change in volume results in a change in the dye concentration and hence an altered fluorescence signal.

Fortunately, most of the above mentioned sources of artefacts are rather small and might be neglected when the observed signals are robust. However, if minute signals are expected or observed, the artefacts are likely to become relevant.

An almost unavoidable artificial situation in live cell imaging is the fact that the RBCs are attached to a (coated or uncoated) coverslip. The only way to exclude artificial conclusions is the comparison/combination with complementary methods.

Last but not least, live cell imaging is often used to detect hormonal or pharmacological stimulation of RBCs. To have a proper control of the solution surrounding the cell, a local perfusion (a micro-manipulator-associated cannula placed close to the RBCs to apply a laminar flow) is preferred over an exchange of the bulk solution of the entire dish that almost certainly would lead to slow gradients of the exchanged solutions and a loss of control concerning the timing of the drug or hormonal stimulation. Because RBCs contain a number of mechanically sensitive proteins[38], one has to make sure that the

flow does not change with the application, and therefore, the flow must be kept constant (also under control conditions) and just the solution composition needs to be switched from the battery of solutions.

3.6. Adhesion force measurements

Adhesion is traditionally measured by either microscopic investigation, quantifying a microscopic aggregation index[87] or by indirect methods based on the properties of RBC suspensions. Such techniques include sedimentation-associated procedures, transmission light or ultrasound scattering, impedance measurements, determination of viscosity or other rheometric methods[88]. The classical methods to measure RBC aggregation have been recently reviewed[89]. However, with regard to adhesion force measurements, rheometric techniques have widely been used[90,91]. These methods are all indirect and suffer from a limited amount of information on the number of cells involved or the impact of RBC morphological and deformability changes.

Quantitative force measurements

Recently, two quantitative RBC intercellular adhesion measurements were introduced at the single-cell level and compared to each other[92,93]. The two techniques are holographic optical tweezers (HOT) and atomic force microscope-based single cell force spectroscopy (SCFS). To exert forces on cells with optical tweezers, a limited force regime is available due to cell damage with increasing laser power, i.e., there exists an upper limit of force at which the adhesion forces between cells can be measured. In addition to that, a lower limit of measurable adhesion forces exists for the SCFS, which is due to both the limited force resolution of the system and the squeezing of the cells during the measurements that can possibly induce adhesion force artefacts (see below). Both limits could be illustrated by measuring the small adhesion forces between single RBCs under physiological conditions[92,93]. In the course of the experiment, a cantilever with an attached cell was lowered onto another cell that was attached to the substrate until a preset defined constant force was reached and kept stationary for a defined contact time (Figure 6A). Subsequently, the cantilever was withdrawn at a constant speed. During the approach and retraction, the cantilever was deflected as a consequence of the acting forces. This deflection, which is proportional to the acting forces, was recorded in force-distance curves (Figure 6C). The retraction curve was typically characterised by the maximum force required to separate the cells from each other, referred to as the maximum unbinding force. Under physiological conditions, RBCs should not adhere at all. Hence, it was expected that a very small force close to zero would be achieved. In contrast, a mean force of approximately 29 pN was measured. To determine if this force was real or an artefact of the SCFS technique itself, HOT measurements were conducted. The HOT exerts a force of 15-20 pN (Figure 6D) at most because a higher laser power would damage the RBC.

Considering the mean adhesion forces of 29 pN derived under control conditions from the SCFS measurements, it should not be possible to separate two RBCs by the use of HOT once they have touched each other. Surprisingly, in the measurements with the optical tweezers, it was possible to easily separate the cells. Even more surprisingly, it

was difficult to detect any adhesion force at all (Figure 6D).
The only way to explain the difference in both techniques is the slightly invasive nature of the SCFS. An inevitable part of the SCFS measurements is the requirement for a preset force set point that is used as a marker if both cells have come into close contact (i.e., squeezing the two cells together with a certain set point force). This invasive squeezing of the cells is artificial, and it most likely induces a small adhesion by itself.
The above mentioned problems should not arise when probing RBCs for specific molecules, e.g., for testing receptor binding[94]. In this case, the cantilever is functionalised with the specific molecules (e.g., fibrinogen) and allows measuring the adhesion between a molecule-coated cantilever and the RBC.

Selection criteria for force measurements
When measuring forces between RBCs, it would be desirable to combine the complementary methods of SCFS and HOT. Unfortunately, both methods are complex and laborious, and this advice might not always be feasible. Therefore, the tool can be chosen according to the dimension of the expected force. The SCFS is advised for adhesion forces larger than 30 pN and the HOT for adhesion forces smaller than 30 pN. While the squeezing of the cells in the SCFS measurements is the critical parameter, the laser power is the critical parameter in the HOT measurements.

4 Conclusions

We are left with the impression that a significant portion of the past literature on RBCs should be re-read to verify whether it could have been affected by the problem of cell contamination. Of course, one will not incur such problems when studying RBCs at a single-cell level.
Recent studies reveal that RBC populations are rather heterogenous[10], as depicted in Figure 4, which may result in additional problems when working with bulk suspensions as well as with single RBCs. A major reason for the inhomogeneities of circulating RBCs are differences in the cell age[95]. There are indications that the plasma membrane Ca^{2+} pump activity decreases with RBC age in a monotonic fashion, which may lead, at least for some cells, to changes in the sodium and potassium content. However, when performing single-cell experiments, the cells are chosen randomly, i.e., cells can be from one or the other end of the age scale. Moreover, variable amounts of circulating reticulocytes also contribute to the variability of measurements performed on bulk RBC suspensions, even after WBCs and platelets have been carefully removed. Therefore, a flow chart of an optimised protocol for studying a property/component of mature RBCs should involve the following: leukodepletion by any suitable method/filter; gelatin zymography to ascertain the level of residual PMNs; the efficient use of anti-proteases; reticulocyte quantification (count and/or Western Blotting of, for instance, CD71, the transferrin receptor); separation of RBCs into subpopulations of different density/age and isolation of a population of “mature” RBCs[95]. However, the availability of a reliable and artefact-free separation technique is still debated.
Alternatively, to elucidate the inter-cellular variability of responses, measurements in cell

suspensions should be combined with singe-cell techniques such as fluorescent live cell imaging, FCM and/or patch-clamp approaches. However, even between single-cell techniques, there are regularly discrepancies and confusing interpretations because cell behaviour is highly sensitive, and often the devil is in the experimental details. Therefore, considerations that will lead to better harmonisation of experimental conditions are timely and relevant, especially regarding the accumulation of large amounts of data in the literature.

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Figure legends

Figure 1: (**A**) Gelatin zymography of the pelleted cell fraction obtained by the "Accuspin™ System-Histopaque®-1077" method (Sigma-Aldrich, Inc., St. Louis, MO, USA) used in a recent work for isolating RBCs in supposedly pure form[12]. As per the manufacturer's instructions, a blood sample was freshly drawn in 0.1 volumes of 3.8% (w/v) tri-sodium citrate as the anticoagulant and immediately layered in the Accuspin™ tube. After centrifugation, the RBC pellet was collected (after removal of the septum from the Accuspin™ tube), washed two times in ten volumes of PBS and processed for gelatin zymography, as previously described[14]. The arrows indicate the bands corresponding to the typical pattern of in-gel digestion of gelatin by the granulocytic pro-metalloproteinase 9 and its higher molecular weight aggregate with lipocalin and are indicative of the presence of PMNs in the RBC fractions. The lane marked "T" corresponds to unseparated blood cells. The lane marked "F" contains a sample of RBCs that was passed through a cellulose filter to remove leukocytes and platelets and is indeed free of PMNs. The pellet of supposedly pure RBCs resulting from the Accuspin™ separation contains almost all of the PMNs that were present in the unseparated blood ("AH"). The lane marked "H" is a sample of RBCs obtained by density centrifugation in the Histopaque®-1077 solution but in a standard tube (not Accuspin™). In each lane, the equivalent of 10^7 cells was loaded. In the lane "PMNs", $2x10^2$ pure PMNs were loaded. (**B**) Contaminating PMNs are carried all along during the preparation of ghost membranes, and they ended up in the final sample of supposedly pure ghosts. PMNs were detected using gelatin zymography[14] in ghosts prepared from washed ("W") or filtered ("F") RBCs with the method of Dodge[96] as previously detailed[5], but with the omission of phenyl-methyl-sulphonyl-fluoride to show the consequences of proteolysis. Where proteolysis was inhibited, diisopropylfluorophosphate (DFP) was used at a final concentration of 1 mM in PBS, to pre-treat RBCs for 5 min at 25 °C before hypotonic haemolysis to prepare the ghosts. During ghost preparation, at the end of the third sedimentation, the "button" of dark, thick material that forms at the bottom of the ghosts and that is especially visible in samples where contamination by PMNs is high was either removed or left in place ("button" - or +). After preparation, the ghost suspension was brought to the volume of the original packed RBCs and processed for gelatin zymography as described[14]. In each lane, the equivalent of 10^7 RBCs was loaded. Pure PMNs were loaded as a standard in the adjacent lanes, in the numbers given. (**C**) The ghosts prepared in the various modes described above were processed for SDS-

PAGE and Western blotting. Analysis of ankyrin and protein 4.1 revealed that both proteins were quantitatively decreased when the ghosts were prepared from filtered or washed RBCs in the absence of DFP. When the “button” of debris was not removed, both proteins were heavily damaged.

Figure 2: (**A**) Changes in morphology and in the intracellular Ca^{2+} levels in RBCs from a sickle cell patient caused by inhibition of the Ca^{2+} pump. RBCs from a sickle cell disease patient suspended in isotonic medium containing 1.8 mM $CaCl_2$, 10 mM glucose and 0.1% bovine serum albumin. The cells were loaded with 10 μM Fluo-4 AM for 40 min in the presence or absence of 5 mM Na-orthovanadate (ov). The upper panels show bright field images of control and orthovanadate-treated cells, and the lower panels represent the corresponding readouts of Fluo-4 fluorescence. Extensive vesiculation, cell disintegration and echinocytosis followed uncontrolled Ca^{2+} loading. (**B**) RBCs from two healthy subjects (P1 and P2) were collected, filtered and stored for 1-21 days in Ca^{2+}-free citrate-containing glutathione (GSH) conservation solution, and they were then re-suspended in the incubation medium containing 145 mM NaCl, 4 mM KCl, 1.8 mM Ca^{2+}, 10 mM glucose and 0.1% bovine serum albumin. The cells of both donors retained normal discocyte morphology over at least one week in the citrate phosphate dextrose solution, and P1 was considered "a better quality donor" during conventional quality control tests. Of importance are the inter-individual differences in the responses and acute changes in morphology in the presence of extracellular Ca^{2+} and the progressive deterioration of cellular quality associated with ATP and GSH depletion and changes in the ion and water content.

Figure 3: Electrical model of a patch-clamp for cell-attached (**A**) and whole-cell (**B**) configurations. (**A**) When narrowing the tip of the pipette, its resistance R_{pip} is increased. Adaptation of the shape of the pipettes for RBCs (especially human RBCs) has led to the use of pipettes with an R_{pip} between 10 and 15 MΩ. Up to 80% of seal attempts are successful with such pipettes, depending on the solutions used. These values are not a problem for single channel studies, as they remain well below the patch resistance R_{patch} through which single channel currents are recorded and these currents can therefore be easily recorded. However, as suggested by Barry and Lynch, distortion of the potential that is applied to the membrane can occur in small cells in relation to the global membrane resistance of the cell R_m [97]. This resistance in small cells can be on the order of magnitude of several GΩ, which is not much lower than R_{patch}. Then, variations in the pipette potential can induce changes in the global membrane potential, and this would result in large errors in the estimation of the single-channel conductance and of the reversal potential. Barry and Lynch[97] conclude their work by revealing the distortion between the apparent and real conductance of the single channel conductance: $\gamma_c=\gamma_{app}(1+R_m/R_{patch})$, where γ_c and γ_{app} are the real and apparent conductance of the channel, respectively. This clearly highlights the possible distortion of conductance estimation with cells showing a high membrane resistance. (**B**) For whole-cell experiments, two resistances are used in parallel in the electrical model: the global membrane resistance R_m and the seal resistance R_{seal}. The complexity when using this configuration with small cells is linked to the possibly high value of membrane resistance.

The seal resistance is barely higher than 100 GΩ, and in small cells, such as RBCs, the global resistance can reach this order of magnitude[98]. Then, the contribution of the seal leak to the currents recorded in this configuration is not negligible: the current measured between the two electrodes can be attributed either to the membrane current or a seal leak, and the voltage applied to the pipette might be different from the voltage that actually occurs at the membrane level. This can induce an underestimation of the channel conductance and a discrepancy between the real and apparent reversal potentials in cell-attached experiments as well as an incorrect global conductance and a shift in the I/V curves in whole-cell experiments.

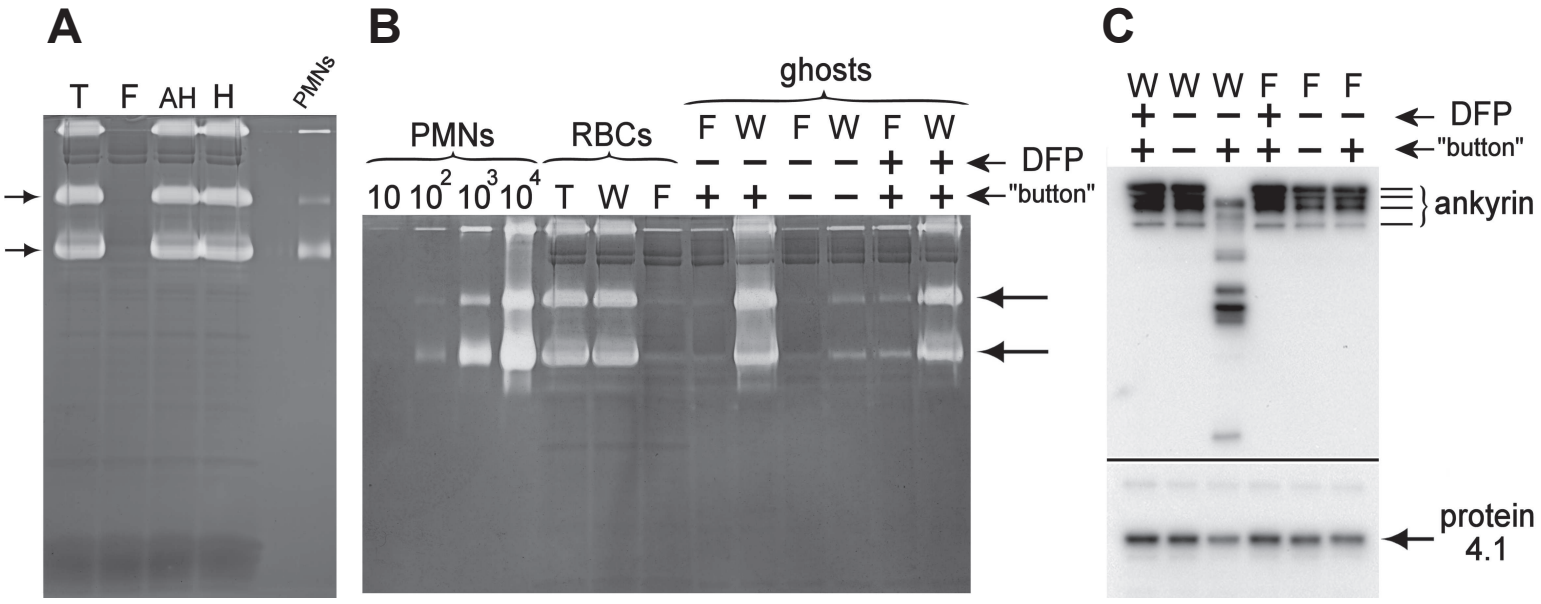
Figure 4: Heterogeneity of RBCs - measurements derived from automated patch-clamp whole cell recordings (NPC-16 Patchliner, Nanion, Munich, Germany). (**A**) depicts the voltage protocol. The bath solution contained 80 mM NaCl, 3 mM KCl, 10 mM $MgCl_2$, 35 mM $CaCl_2$, and 10 mM HEPES /NaOH, pH 7.4, while the pipette solution contained 50 mM CsCl, 10 mM NaCl, 60 mM CsF, 20 mM EGTA, 10 mM HEPES /CsOH, pH 7.2. The seal resistance was in the range of 1-5 GΩ at a holding potential of -40 mV. (**B**) exemplifies the recordings from single RBCs of 2 healthy subjects, (a) and (b). The current traces are superpositions of leak currents and channel-mediated currents. The non-Ohmic appearance of the currents indicates to differences in the cellular properties rather than in the patch resistance. Panel (**C**) shows the I/V plots for the whole cell currents (mean ± SD, n=24 cells per subject). Please note that both samples were from freshly drawn RBCs (experiments within 2 h) and are not attributed to different cell treatments as outlined in Figure 2.

Figure 5: Fluorescence spectra of the Ca^{2+} fluorophores Indo-1 and Fluo-4 as well as the excitation spectra of Fura-2. The normalised fluorescence intensity is plotted against the wavelength and the Ca^{2+} concentration. For each dye (rows), the spectra were measured without haemoglobin, with 0.034 g/dl and with 0.34 g/dl haemoglobin (columns). The arrow points to the heavy spectral alteration of the Indo-1 spectrum in the presence of 0.034 g/dl haemoglobin. Additionally, the Fura-2 spectrum is altered in a way that does not allow a valid ratiometric read-out. Fluo-4 fluorescence is suppressed, but it seems to be the best choice for measurements in RBCs due to its high dynamic range[99]. This figure is a reprint from the original publication Kaestner et al. 2004{Kaestner:2005he}.

Figure 6: Panel (**A**) shows a sketch of the working principle of single-cell force spectroscopy (SCFS). A cell is bound to a cantilever and is brought into contact with another cell at the surface. During the approach and withdrawal of the cell, the deflection is monitored and gives direct information about the adhesion force between the cells. Panel (**B**) shows a sketch of the working principle of the optical tweezers measurements. Two RBCs are trapped in the foci of two laser beams and are brought into contact. By measuring the deflection of the cells out of the centre of the laser foci, one can determine the adhesion force between the cells. Panel (**C**) shows a force vs. distance curve derived from the SSFS measurements. A weak interaction of approximately 20 pN can be observed that is only due to an artefact of the measurement (see text). This 20 pN is the lower limit that one can measure using this type of cell with this technique.

Panel (**D**) shows a force calibration of one RBC in an optical trap. It can be observed that with the given laser power, the trap is only linear up to forces of 15 pN, i.e., this is the upper limit that can be measured with this technique on these types of cells. Panel (**A**) is a reprint of the original publication by Steffen et al. 2011[92], and panels (**B**) and (**D**) are reprints from the original publication by Kaestner et al. 2011[93].

Figure 1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B
ghosts
PMNs RBCs F W F W F W
DFP
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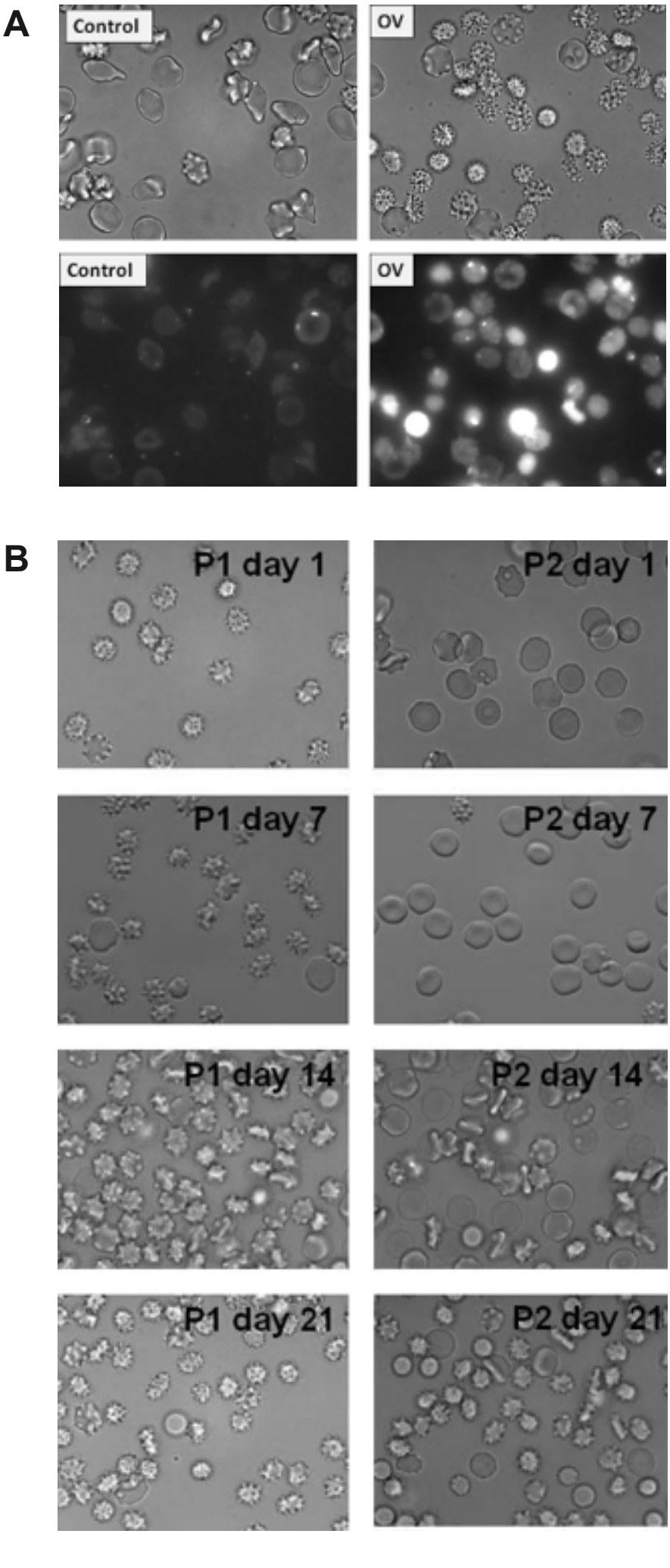
C
W W W F F F
DFP
"button"
ankyrin
protein 4.1

Figure 2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OV
Control
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B
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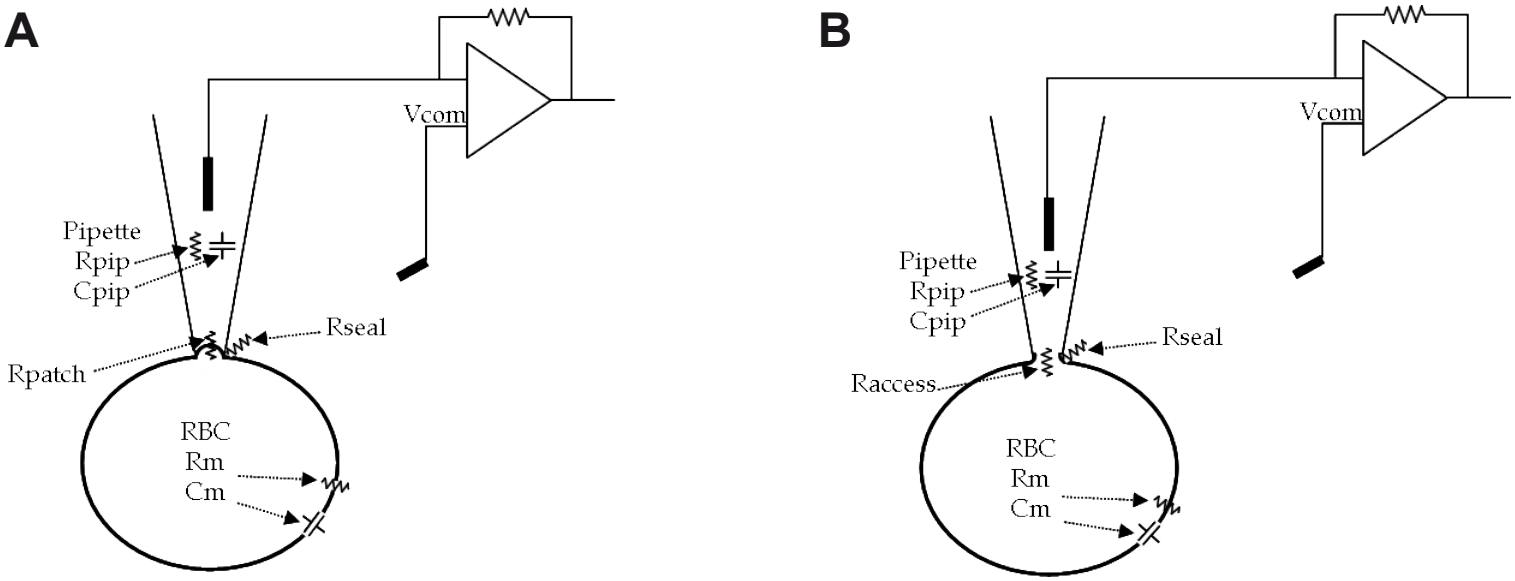
Figure 3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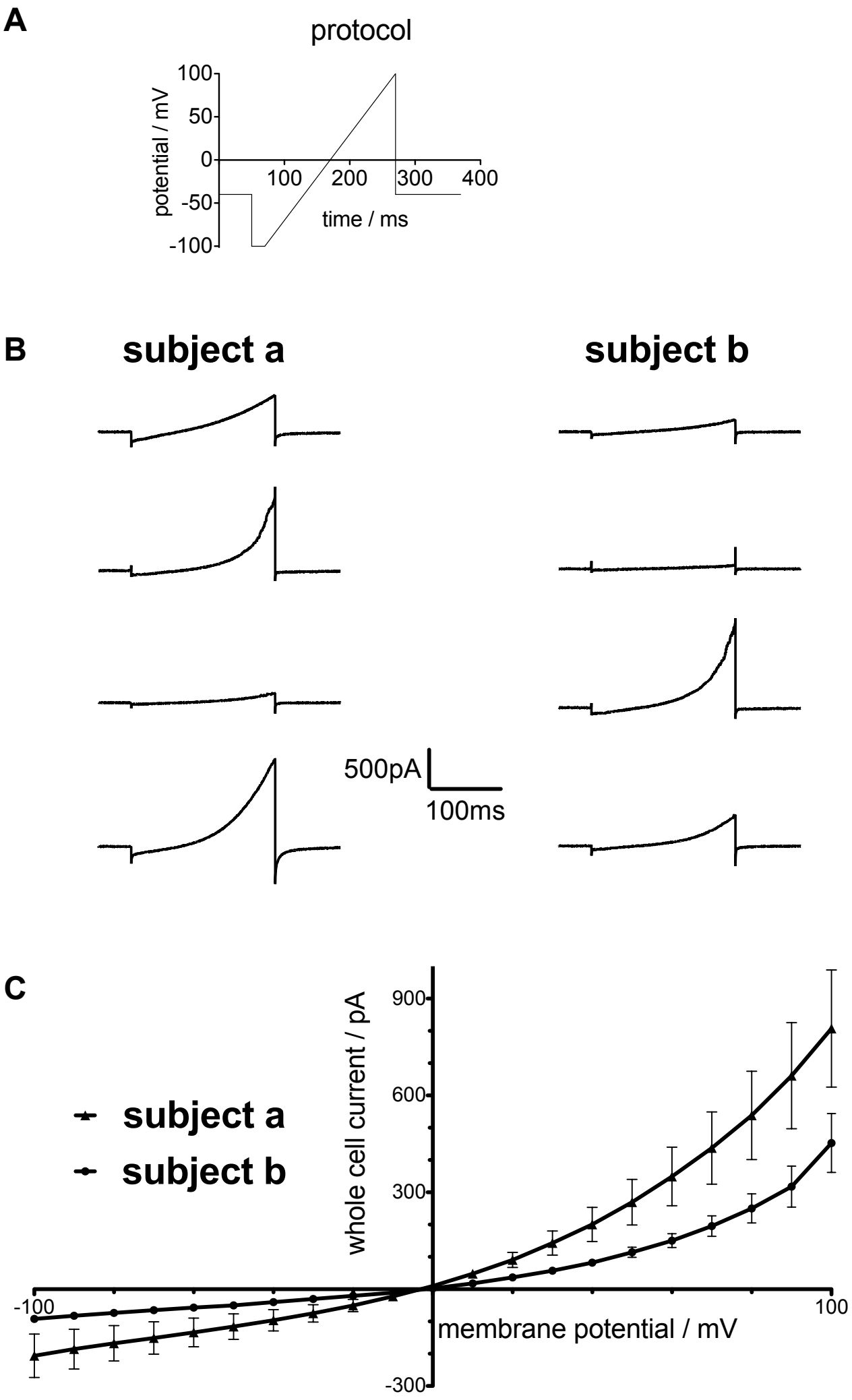
Raccess
RBC
Rm
Cm

Figure 4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50
0
-50
-100
100
200
300
400
time / ms
B
subject a
subject b
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100ms
C
subject a
subject b
whole cell current / pA
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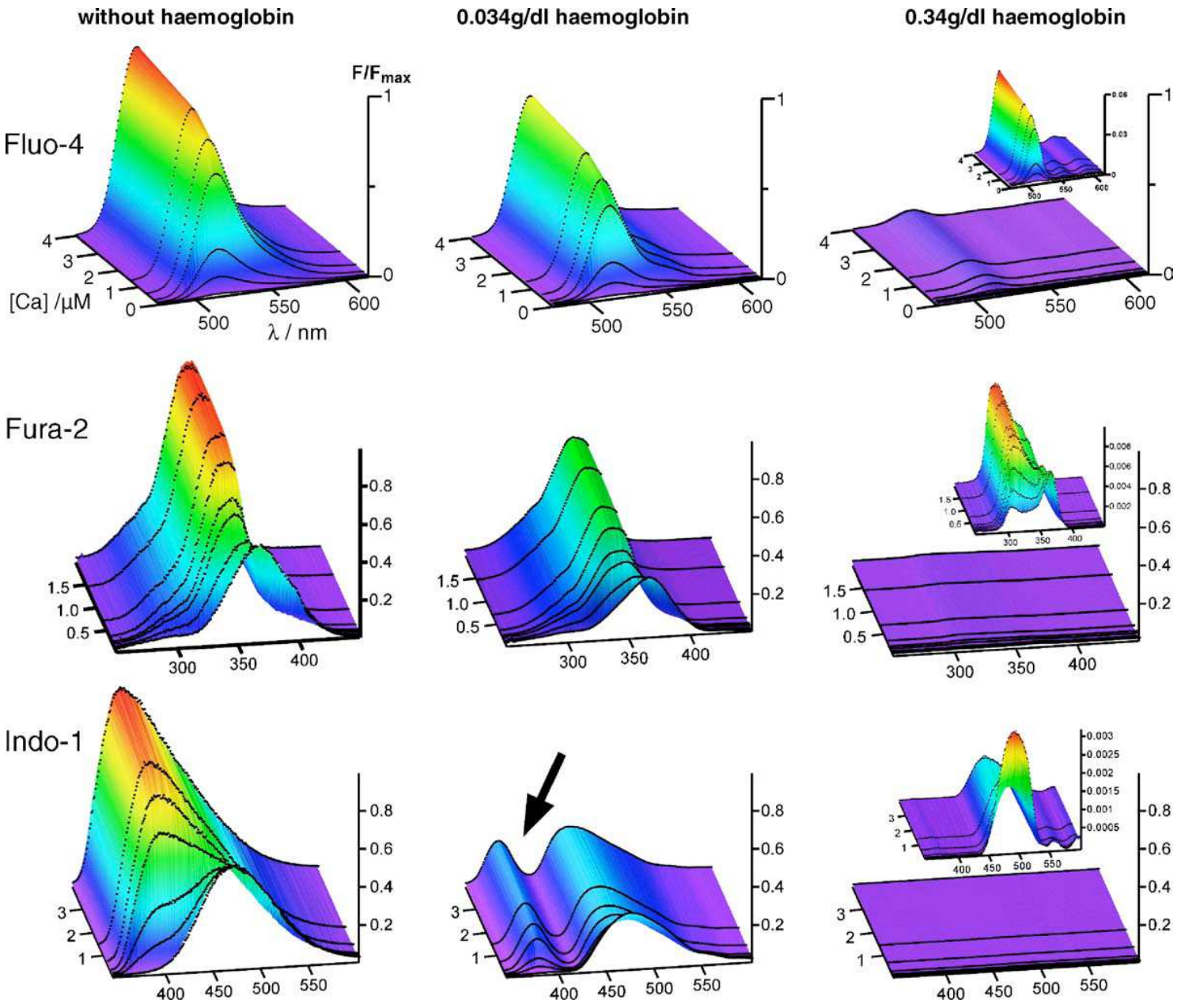
600
300
-300
-100
100
membrane potential / mV

Figure 5


without haemoglobin
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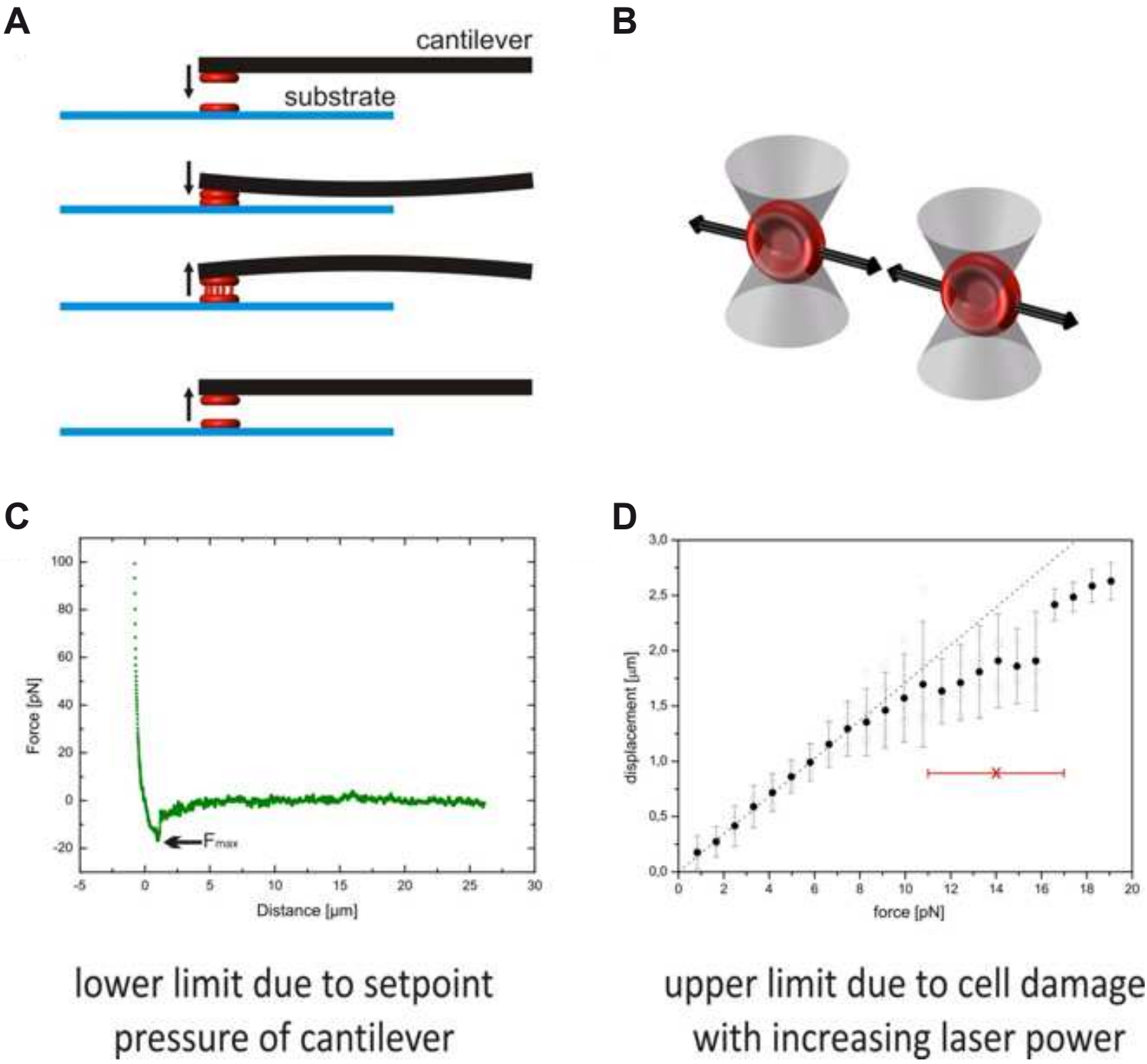
0.34g/dl haemoglobin
Fluo-4
Fura-2
Indo-1
F/F_max
[Ca] /µM
λ / nm

Figure 6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cantilever
substrate
B
C
Force [pN]
Distance [µm]
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D
displacement [µm]
force [pN]
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upper limit due to cell damage with increasing laser power